

Neocortical Cell Migration: GABAergic Neurons and Cells in Layers I and VI Move in a Cyclin-Dependent Kinase 5-Independent Manner

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The adult mammalian cerebral cortex arises from a complex series of neuronal migrations. The primitive layer known as the preplate is split into an outer marginal zone and an inner subplate by invading cortical plate neurons in an “inside-out” pattern of layering with respect to time of neuronal origin. In cyclin-dependent kinase 5 (Cdk5)-deficient mice (*cdk5*^{-/-}), the earliest born cortical neurons split the preplate, but later born neurons arrest below the subplate, resulting in an ectopic “outside-in” layer of neurons normally destined for layers II–V. We have pursued this analysis in *cdk5*^{-/-} ↔ wild-type chimeric mice coupled with experiments in cell culture. *In vitro* migration assays show no difference in migrational ability between embryonic *cdk5*^{-/-} and wild-type neurons. In *cdk5*^{-/-} chimeras, layers I and VI are made up of both mutant and wild-type

genotype neurons, whereas layers II–V contain predominantly wild-type cells. In addition, a thin layer of neurons is found below layer VI, made up of *cdk5*^{-/-} cells; bromodeoxyuridine labeling suggests that these neurons were destined for layers II–V. Scattered *cdk5*^{-/-} cells are found throughout layers II–V, but these neurons are always found to be GABAergic. The findings suggest that Cdk5 is not required for migration of either the deepest cortical plate neurons or the GABAergic neurons from the ganglionic eminences. The migration of layer II–V pyramidal neurons, however, is intrinsically blocked by Cdk5 deficiency, thus suggesting that different neuronal cell types use distinct mechanisms of migration.

Key words: tangential migration; radial migration; chimeras; cyclin-dependent kinase 5; BrdU; cortical cell culture

The cerebral cortex begins as a single layer of pseudostratified neuroepithelial cells and develops into a six-layered laminar structure (Boulder Committee, 1970). First the preplate, a layer consisting primarily of transient populations, forms. The preplate is split into two layers by radially migrating neurons destined to form the true cortical plate (Marin-Padilla, 1978). The first cohort of arriving neurons is followed by subsequent groups of cells that migrate past the subplate, past earlier born cortical plate neurons, and stop just under the marginal zone. The end result is a cerebral cortex that is born inside-out (Angevine and Sidman, 1961). Tangential migration of GABAergic interneurons from their ventricular origin in the ganglionic eminences adds additional complexity to the process (Anderson et al., 1997; Tamamaki et al., 1997).

The range of gene products whose coordinate expression is required during corticogenesis can be appreciated by considering the genetic defects, in both humans and rodents, that are known to disrupt this process. These mutations include the engineered null alleles of cyclin-dependent kinase 5 (Cdk5; Ohshima et al., 1996; Gilmore et al., 1998) and its activating subunit p35 (Chae et al., 1997; Kwon and Tsai, 1998). Cdk5 has close homology to other cyclin-dependent kinases, but its highest expression and levels of detectable activity are found within postmitotic neurons

(Hellmich et al., 1992; Lew et al., 1992; Tsai et al., 1993). Cdk5-deficient mice develop normally through the point of early cortical plate neurons bisecting the preplate (Gilmore et al., 1998). Later born neuronal cohorts, however, appear unable to migrate past the subplate and form an outside-in layer located beneath the subplate; the mice subsequently die in the perinatal period (Ohshima et al., 1996, 1998, 1999; Gilmore et al., 1998). The proper migration of the earliest cortical plate neurons (layer VI) contrasts with the failure of later born immigrants to layers II–V and suggests that there are important differences between migratory programs of these two populations. This bipartite effect on migratory ability is not observed in *reeler* and related mutations, in which all neocortical neurons reside below the subplate (Falconer, 1951; Caviness and Sidman, 1973; Caviness and Rakic, 1978; Howell et al., 1997; Sheldon et al., 1997; Sheppard and Pearlman, 1997; Ware et al., 1997). Cdk5 may thus have a unique involvement in the migration of layer II–V neurons.

In this study, we demonstrate that *cdk5*^{-/-} neurons are fully competent to migrate in a simple culture model, in a manner indistinguishable from wild-type neurons. In contrast, we demonstrate that the *in vivo* radial migration of neurons in cerebral cortical layers II–V is Cdk5-dependent even in the relatively normal “terrain” of the Cdk5 chimera. Importantly, the tangential migration of the GABAergic interneurons from the ganglionic eminence is found to be Cdk5-independent. These findings imply differential requirements for migration by early and late cerebral cortical precursors, as well as by tangentially migrating GABAergic precursors.

MATERIALS AND METHODS

Migration assays. Cerebellar explant cultures were established from embryonic day 18 (E18) fetuses (plug date being E0). Pregnant *cdk5*^{+/-} dams from *cdk5*^{+/-} matings were killed by cervical dislocation, and their

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embryos were quickly removed into ice-cold HEPES-buffered saline solution. Heads were removed, and bodies were saved for PCR analysis (Ohshima et al., 1996; Gilmore et al., 1998). Tissue was dissected into small pieces, ~50–150 μm in diameter, in chilled HEPES-buffered saline solution. Five to eight pieces of tissue were placed into 24 well plates coated with poly-L-lysine (Sigma, St. Louis, MO) and laminin (Life Technologies, Gaithersburg, MD) containing Neurobasal medium (Life Technologies) supplemented with B-27 (Life Technologies), Penn-Strep at 100 U/ml (Life Technologies), and 2 mM L-glutamine (Life Technologies). Cultures were grown for 1 d before fixation. 4,6-Dimidino-2-phenylindole (DAPI) staining was used to visualize cell nuclei. The location of cells relative to the explant edge was determined with NIH Image 1.6, and distances were calculated with Microsoft (Redmond, WA) Excel 98 software. Because cells do not uniformly migrate away from explants, photographs were taken at varying angles of 0, 180, 270, or 90° (in that order) from the center of the culture. The top of the plate was considered 0°. If no cells migrated away from an explant, it was not used for analysis.

Chimeric mice derived from *cdk5*^{-/-} embryonic stem cells. *cdk5*^{-/-} embryonic stem (ES) cell clones were obtained using the high G418 selection method (Mortensen et al., 1992). *cdk5*^{+/-} ES cells, derived from clone 57 (Ohshima et al., 1996), were cultured in 1 mg/ml G418 (Life Technologies) for 6–7 d. Resistant colonies were picked and genotyped individually as described previously (Ohshima et al., 1996, 1999). To generate chimeric mice, *cdk5*^{-/-} ES cells (57G23) were injected into blastocysts derived from C57BL/6 females mated with males from the ROSA26 transgenic strain. The cells of these animals contain a β -galactosidase transgene driven by a nearly ubiquitous cytomegalovirus promoter. As a result, histologically detectable expression of β -galactosidase is observed in nearly all cells, including neurons (Magrassi and Graziadei, 1996; Zambrowicz et al., 1997). Age-matched nonchimeric littermates, C57BL/6, and C57BL/6 \times ROSA26 mice were used as controls.

Histological and immunohistochemical analysis. Adult brains from chimeras and controls were obtained after transcardial perfusion with 3% paraformaldehyde in 100 mM PIPES, pH 7.4, for 15 min. Brains were dissected and transferred overnight (or longer) to 20% sucrose and PBS at 4°C. For embryonic anti-GABA immunostaining, pregnant dams from *cdk5*^{+/-} matings were killed at E18.5. Embryos were transferred immediately to cold PBS. Heads were transferred to 4% paraformaldehyde in 0.1 M phosphate buffer for immersion fixation overnight at 4°C followed by transfer to 18% sucrose and PBS overnight (or longer) at 4°C. Bodies were retained for PCR genotyping (Ohshima et al., 1996; Gilmore et al., 1998). Sections were cut on a cryostat at 12 μm . All sections were kept at -70°C before use. For 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) studies, coronal sections of forebrain were incubated for 4 hr at 37°C with 1 mg/ml X-gal in 35 mM K₃Fe(CN)₆, 25 mM Fe₃(CN)₆, 2 mM MgCl₂, 0.01% deoxycholic acid, and 0.02% Nonidet P-40 followed by several washes with H₂O. For bromodeoxyuridine (BrdU) immunostaining, sections were acid-permeabilized with 2N HCl for 75 min followed by neutralization with eight washes of PBS of 5 min each.

Immunocytochemistry was performed using primary antibody buffer consisting of PBS with 0.5% Tween 20 and 5% goat serum. The primary antibodies used and their dilutions were as follows: rat anti-BrdU (Accurate Chemicals, Westbury, NY), 1:6; rabbit anti-Cdk5 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:100; rabbit anti-GABA (Sigma), 1:1000; mouse anti-NeuN (A60; a kind gift from Richard Mullen, Woods Hole Oceanographic Institute, Woods Hole, MA), 1:50; and mouse anti-calbindin (Sigma), 1:1000.

Primary antibodies were visualized in PBS and 0.5% Tween 20 with 20% goat serum. The following secondary antibodies were used: goat anti-rat IgG conjugated to Cy3 (Chemicon, Temecula, CA), 1:400; goat anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA), 1:400; and goat anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch), 1:200. Sections were counterstained with 1 $\mu\text{g}/\text{ml}$ DAPI in PBS for 10 min, followed by two washes in PBS for 5 min.

Purkinje cell counts were performed on three nonadjacent midsagittal sections. Cresyl violet staining was performed on coronal sections of forebrain and sagittal sections of hindbrain according to standard protocols. Cerebral cortical neurons were counted under 400 \times magnification in a field ~600 μm in diameter for Cdk5 and NeuN double staining. For GABAergic staining, cells were examined with both light and fluorescent techniques and scored as β -galactosidase (β -gal)-positive if they contained an X-gal deposit within or immediately adjacent to the neuron

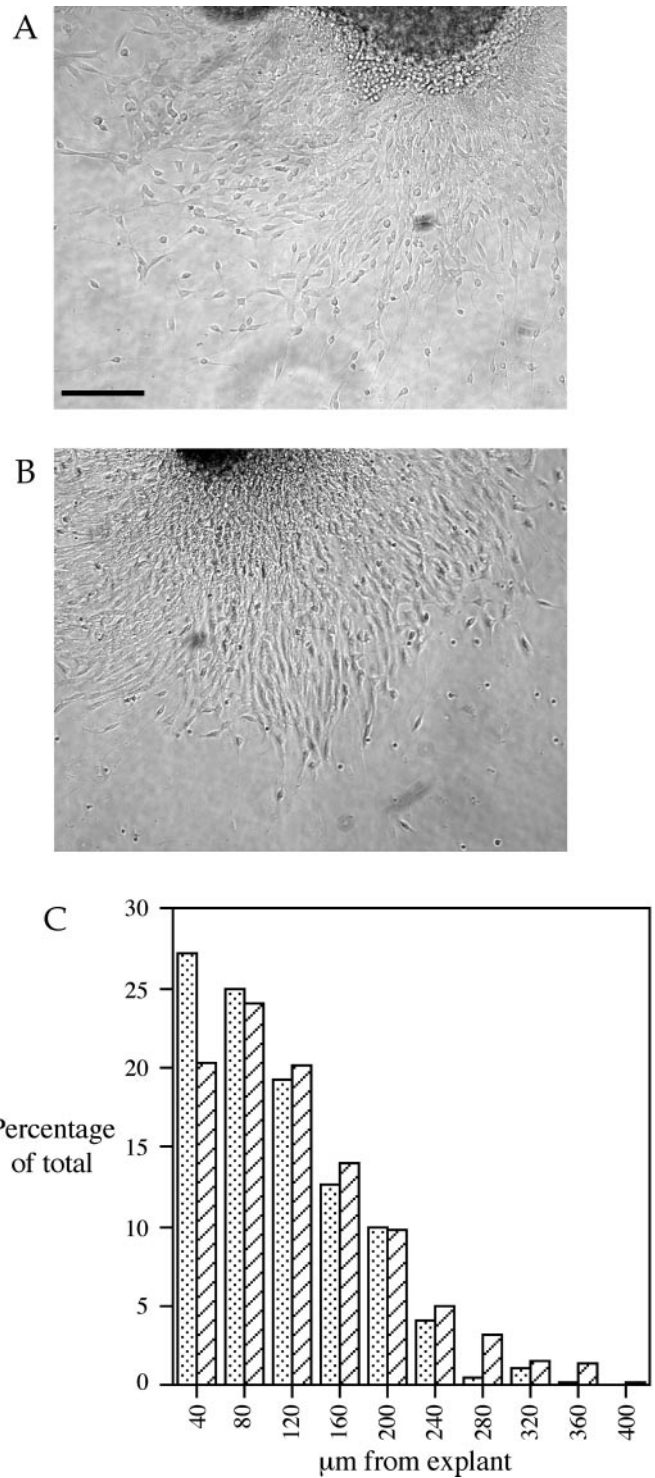


Figure 1. *In vitro* migration of cerebellar neurons on poly-L-lysine and laminin after 1 d. Shown here are the cell migration patterns from explants of wild-type (*A*) and *cdk5*^{-/-} (*B*) embryonic cerebellum. Processes consisting of radial glia and neurons are extended from the explants, and numerous migrating cells can be seen. No qualitative differences are seen between the two genotypes. *C*, Extent of migration represented as a frequency histogram of the distance migrated by granule cells from explant. The y-axis represents the relative percentage of neurons that traveled a particular range of distances. The x-axis represents the distance traveled from explant. The data for wild-type mice are represented by stippled bars; *cdk5*^{-/-} mice are represented by cross-hatched bars. No difference is seen between the genotypes. Scale bar, 100 μm .

Table 1. Purkinje cell counts determine percentage of wild-type cells in Cdk5 chimeras

Mouse	Wild type	423A	423B	423C	423D	423E	Wild type	326B
Age	Adult	Adult	Adult	Adult	Adult	Adult	P12	P12
Counts	671 ± 23	454 ± 36	637 ± 32	632 ± 38	458 ± 49	508 ± 26	441 ± 40	263 ± 43
Percent wild type	100 ± 5%	68 ± 5%	95 ± 5%	94 ± 6%	68 ± 7%	76 ± 4%	100 ± 9%	60 ± 10%

Purkinje cell counts from single midsagittal sections determine relative percentage of chimerism. All Purkinje cell profiles visible in single sections of wild-type mice (C57BL/6) and six chimeric mice were counted. Average counts are listed along with SDs determined by counts of three nonadjacent sections. The percent wild-type contribution and SD of an individual mouse was determined as a percentage of the Purkinje cell number of the age-matched wild type.

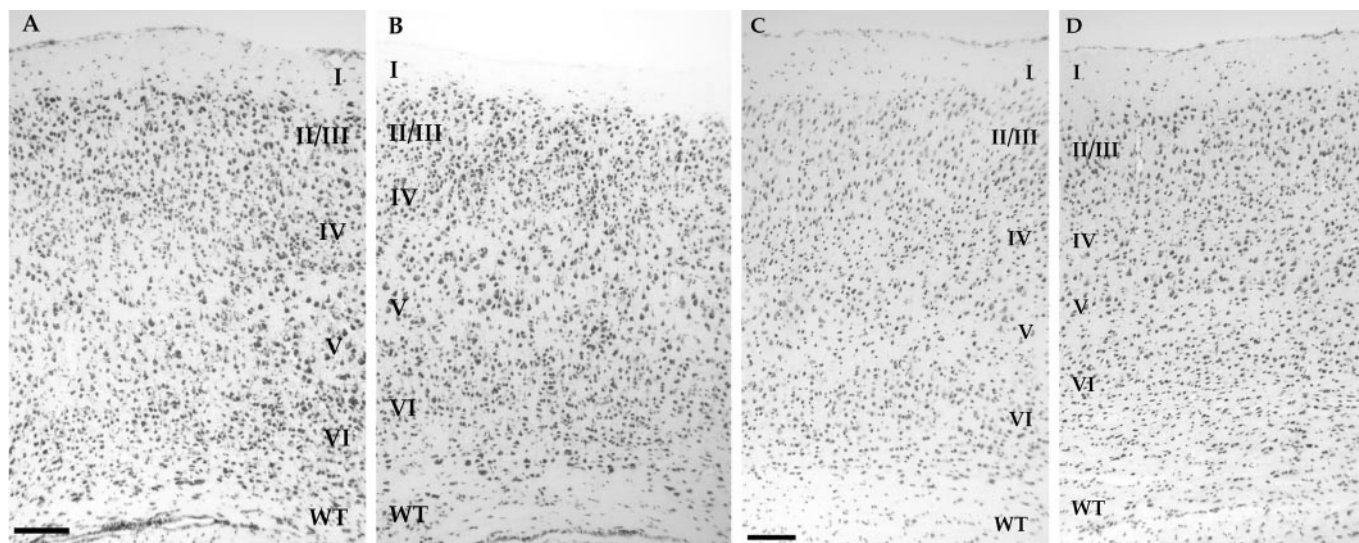


Figure 2. Cresyl violet-stained coronal sections of cerebral cortex. Both developing and mature cortex are illustrated here. *A*, Postnatal day 12 wild type; *B*, Cdk5 chimera 326B; *C*, adult wild type; *D*, chimera 423D. Cortical layers are numbered I–VI with the white matter tract (WT) labeled along the ventral surface. Although different regions of cerebral cortex demonstrate variation in both composition and density of various layers of cells, the basic pattern is present in both wild-type and chimeric mice from early developmental times. Scale bar, 100 μ m.

(see examples in Fig. 6). The boundaries of the six cortical layers were determined with DAPI or NeuN.

All sections were examined by standard light and fluorescent techniques on a Leitz (Wetzlar, Germany) Orthoplan microscope. Images were captured with a Spot digital camera model 1.3.0 from Diagnostic Instruments Inc.

Statistics. SD and Student's *t* tests were performed using Microsoft Excel 98 software. The α value for the Student's *t* test was always 0.05.

RESULTS

The use of chimeric mice has greatly aided the study of many neurological mutations. Our laboratory has previously used this technique to analyze the developmental potential of the cerebellar cells in Cdk5-deficient mice. All *cdk5*^{-/-} Purkinje cells are blocked in their migration. This is true whether they are located in the mutant or in association with wild-type neighbors as in *cdk5*^{-/-} \leftrightarrow *cdk5*^{+/+} chimeric mice (Cdk5 chimeras). In contrast, all genetically wild-type Purkinje cells migrate normally even in the chimera in the presence of mutant neighbors (Ohshima et al., 1999). There are also cell-autonomous defects in cerebellar granule cell migration, but these defects are not complete. Some *cdk5*^{-/-} granule cells are capable of migrating from the external to the internal granule cell layer despite their mutant genotype. The cells of the deep cerebellar nuclei appear to migrate successfully regardless of their *cdk5* genotype (Ohshima et al., 1999).

Cell migration assays

We have shown that Cdk5 is required for proper migration of a number of neuronal progenitors *in vivo* (Gilmore et al., 1998;

Ohshima et al., 1999). The cell biological nature of this requirement, however, is not clear. To test for a potential defect in the general locomotive ability of mutant neurons, explants from the cerebella of E18 embryos derived from the mating of *cdk5*^{+/-} mice were grown in culture for 1 d before fixation. During this time, extensive neurite outgrowth and glial migration occurred along with significant neuronal migration away from the explants. There was no apparent difference between the number of granule cells that had migrated away from the explants in wild-type (Fig. 1*A*) or *cdk5*^{-/-} (Fig. 1*B*) cultures. To estimate the rates at which the nerve cells migrate *in vitro*, their distance from the nearest edge of the explant after 1 d in culture was calculated. The results are shown in Figure 1*C*, and they illustrate that there was no difference between the genotypes in the distance traveled by individual neurons.

Cerebral cortex of chimeras

We have demonstrated previously that migrating cerebral cortical precursors in *cdk5*^{-/-} mice migrate and properly split the preplate, but later born cerebral cortical precursors stall in their migration below the newly formed subplate (Gilmore et al., 1998). The perinatal death of the mutant embryos precludes the study of any late developmental event, including the completion of cortical cell migration. To address this, we generated *cdk5*^{-/-} \leftrightarrow *cdk5*^{+/+} chimeric mice. Cdk5 chimeras survive into adulthood because of the presence of the wild-type cells, yet because all cell populations of these animals are made up mixtures of *cdk5*^{+/+} and

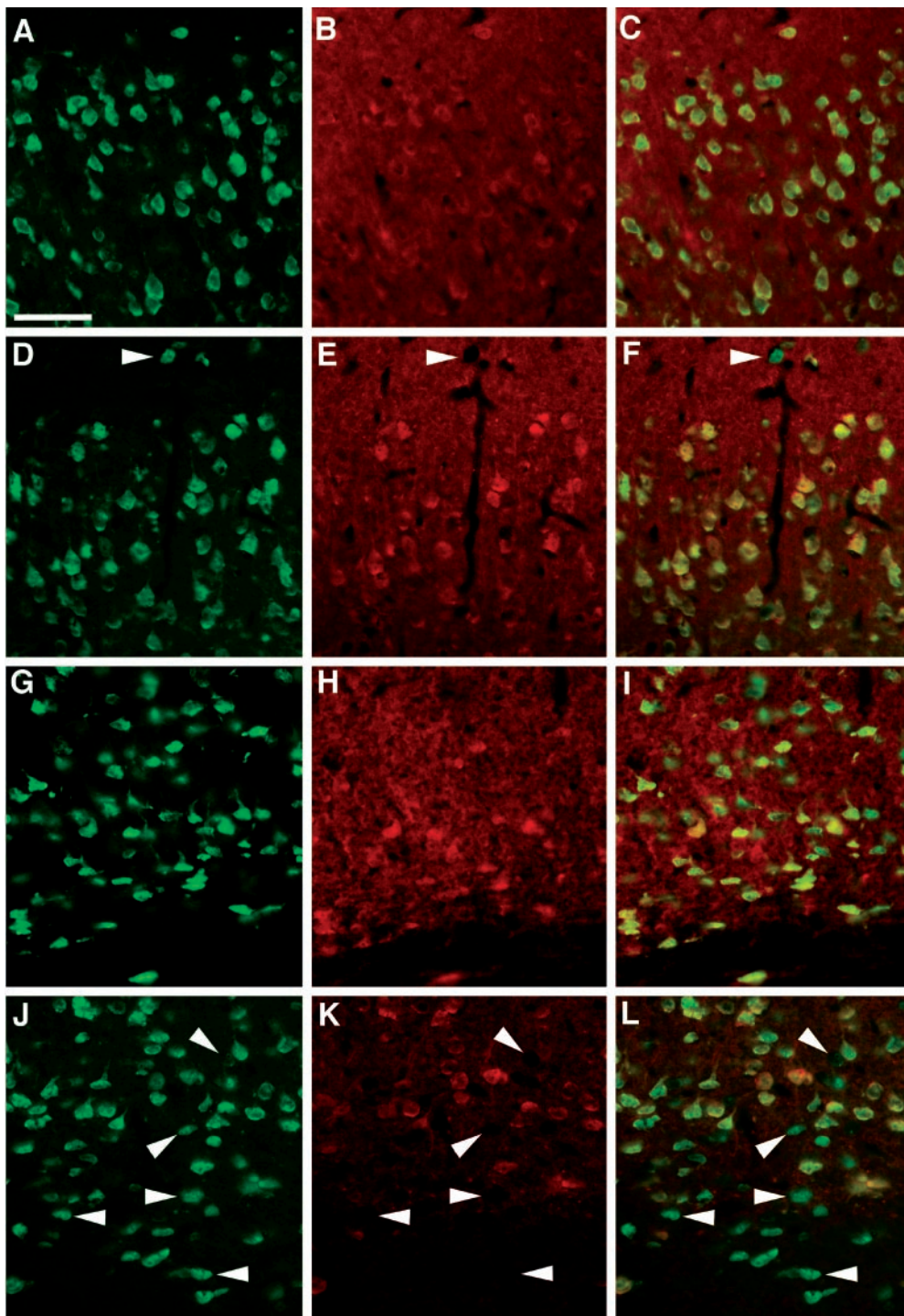


Figure 3. Immunostaining of cerebral cortex from wild-type mice (*A–C*, superficial; *G–I*, deep) and *Cdk5* chimeric mice (*D–F*, superficial; *J–L*, deep). *Cdk5* immunostaining is shown in red (Cy3 secondary; *B*, *E*, *H*, *K*); the neuron-specific marker NeuN is shown in green (FITC secondary; *A*, *D*, *G*, *J*). *C*, *F*, *I*, *L*, digitally combined images. In layers II and III of both wild-type animals (*A–C*) and chimeric animals, e.g., 426D (*D–F*), most neurons are wild-type and thus *Cdk5*-positive. Double staining with NeuN antibody (green) demonstrates that nearly all of the neurons are *Cdk5*-positive, resulting in yellow when combined. There are exceptions to this situation, however. The arrowhead in layer I of the *Cdk5* chimera (*D–F*) demonstrates a single *Cdk5*^{-/-} neuron within layer I. In deeper layers of cerebral cortex in wild-type animals (*G–I*), all layer VI cells contain *Cdk5*. In contrast, neurons in the deeper regions of *Cdk5* chimeras are often found to be *Cdk5*^{-/-}. The arrowheads in *J–L* mark *Cdk5*^{-/-} neurons within the deep portions of layer IV. The white matter tract contains occasional *Cdk5*^{+/+} neurons (yellow) in both wild-type and *Cdk5* chimeras. Scale bar, 50 μ m.

Cdk5^{-/-} cells, the resulting phenotype is both complex and highly informative. ES cells with both copies of the *Cdk5* gene disrupted were injected into ROSA26 blastocysts. The neurons that descend from the cells of the host blastocysts will be wild type at the *Cdk5* locus and, because of their ROSA genotype, each will express bacterial β -galactosidase (Magrassi and Graziadei, 1996; Zambrowicz et al., 1997). This genetic combination allows mutant and wild-type neurons to be distinguished on a cell-by-cell basis by two independent methods: wild-type neurons will express both *Cdk5* and β -galactosidase, whereas mutant neurons will express neither.

The interpretation of the findings in chimeras such as these depends on the ability to determine the ratio of cells of the two genotypes. Because both the host embryos and ES cells were agouti, coat color could not be used. Instead we first examined the cerebellar parenchyma for misplaced Purkinje cells using calbindin staining to help identify the ectopic cells (results not shown) (Ohshima et al., 1999). To quantitate the degree of chimerism, we performed midline Purkinje cell counts of all animals. Within a chimera, the percentage of wild type in one tissue (i.e., cerebellum) closely follows the ratio of the genotypes in the rest of the animal (Soriano and Jaenisch, 1986). In addition, in the Purkinje

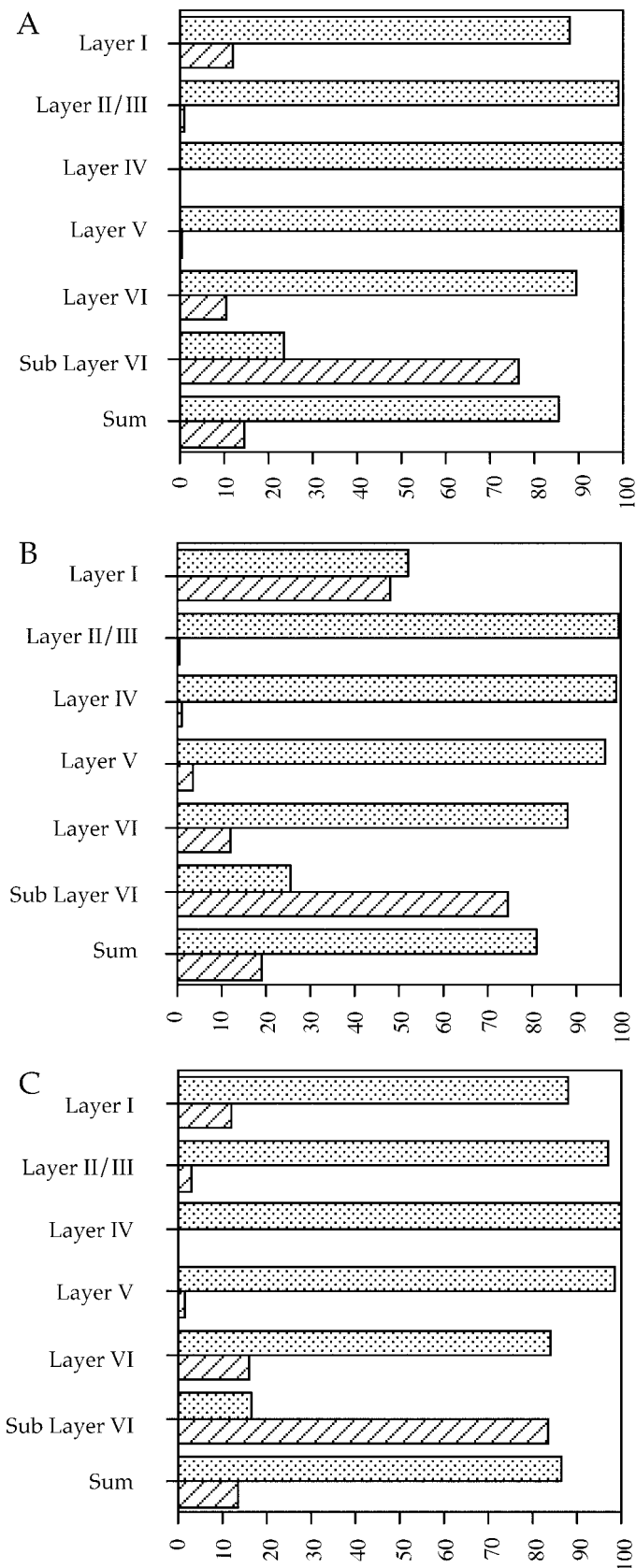


Figure 4. Graphic representation of data in Table 2. Results from chimera 423A are shown in *A*, chimera 423D in *B*, and chimera 326B in *C*. Stippled bars represent the percentage of wild-type neurons within that particular layer of cerebral cortex; cross-hatched bars represent the per-

centage of *cdk5*^{-/-} neurons. *Sum* is all of the neurons from the various layers tallied together. Note the abundance of Cdk5-deficient neurons in Layer I, Layer IV, and Sub Layer VI compared with their near absence from the other layers. Note also that the number of *cdk5*^{-/-} neurons within a layer is never zero.

cell population, there is a fairly homogeneous distribution of the cells of the two genotypes across the two dimensions of the Purkinje cell layer (Mullen, 1977; Herrup and Sunter, 1987). Four Cdk5 chimeric mice were generated with estimated degrees of chimerism ranging from 60 to 76% wild type. Two potential chimeras, 426B and 426C, had no ectopic *cdk5*^{-/-} Purkinje cells, indicating that they had essentially no *cdk5*^{-/-} contribution, and we have used them as wild-type, age-matched, littermate controls (Table 1). Gross histological examination of Cdk5 chimeras indicated that the cytoarchitecture of their cerebral cortices were not dramatically different from wild-type (Fig. 2). Such variation as can be seen in Figure 2 cannot be distinguished from differences that might be expected on the basis of differences in either the anteroposterior or mediolateral location. Examination of animals at young postnatal (Fig. 2*A,B*) and adult (Fig. 2*C,D*) ages revealed similar cellular composition and density in both wild-type (Fig. 2*A,C*) and chimeric (Fig. 2*B,D*) mice; both contain the typical six-layer neocortical architecture. However, the density of cells in the subcortical white matter appears greater in the chimeras. This results in an apparent increase in the thickness of layer VI (Fig. 2*B,D*) compared with wild type (Fig. 2*A,C*).

Most of the chimeric cerebral cortex appears to be populated extensively by wild-type neurons, whereas most of the *cdk5*^{-/-} neurons are located below cortical layer V. Cdk5 is an abundant antigen that is highly expressed in all neurons, a fact that has been exploited previously in examination of the cerebellum of Cdk5 chimeras (Ohshima et al., 1999). In cerebral cortex of wild-type animals, all cells stained positive for the neuron-specific marker NeuN (Mullen et al., 1992) (Fig. 3*A,G*, green) and Cdk5 (Fig. 3*B,H*, red) and thus appear yellow when the two images are combined (Fig. 3*C,I*). A much different pattern is seen in Cdk5 chimeras. Layers II and III contain nearly all wild-type neurons, as indicated by the presence of both NeuN (Fig. 3*D*, green) and Cdk5 (Fig. 3*E*, red, *F*, yellow when photocombined), with a similar composition found in layers IV and V (results not shown). Layer I, on the other hand, contains a substantial number of *cdk5*^{-/-} neurons (white arrowhead) demonstrated by the presence of anti-NeuN (Fig. 3*D*, green) and the absence of Cdk5 (Fig. 3*E*, red) resulting in a green appearance when photocombined (Fig. 3*F*). The *cdk5*^{-/-} neurons in layer VI have a similar staining pattern (Fig. 3*J-L*, white arrowheads). In addition, a substantial number of Cdk5-deficient neurons are found in deep portions of cerebral cortex, primarily in the white matter tract below layer VI (Fig. 3*J-L*, bottom-most arrowheads). These observations suggest that wild-type neurons are capable of proper migration within a chimeric environment, whereas most of the *cdk5*^{-/-} neurons are not. In every chimera we examined, however, there were always a few *cdk5*^{-/-} neurons in layers II–V and many within layer I. To quantitate these observations, the genotype ratios of the different neuronal layers were determined by profile counts of NeuN-labeled cells. The raw counts are presented in Table 2, and the relative percentage of the genotypes in each layer is presented graphically in Figure 4. The essentially wild-type chimeras 423B

←

percentage of *cdk5*^{-/-} neurons. *Sum* is all of the neurons from the various layers tallied together. Note the abundance of Cdk5-deficient neurons in Layer I, Layer IV, and Sub Layer VI compared with their near absence from the other layers. Note also that the number of *cdk5*^{-/-} neurons within a layer is never zero.

Table 2. Distribution of wild-type and *cdk5*^{-/-} cells in cerebral cortex of chimeric mice

Mouse Ge- notype	423A		423D		326B	
	<i>cdk5</i> ^{-/-}	<i>cdk5</i> ^{+/+}	<i>cdk5</i> ^{-/-}	<i>cdk5</i> ^{+/+}	<i>cdk5</i> ^{-/-}	<i>cdk5</i> ^{+/+}
Layer I	4	30	11	12	3	22
Layers II/III	3	240	1	196	9	305
Layer IV	0	123	1	128	0	137
Layer V	1	155	5	130	3	171
Layer VI	28	241	28	202	47	245
Sublayer VI	104	32	120	41	76	15
Total	140	821	166	709	138	895

Cell genotype was determined from double immunostaining with anti-Cdk5 and anti-NeuN, whereas location was determined from NeuN staining pattern. Most *cdk5*^{-/-} neurons are located either within the white matter tract (sublayer VI) or at the bottom of layer VI of the cerebral cortex. These results are graphically represented in Figure 3. Cells were counted under high magnification as described in Materials and Methods.

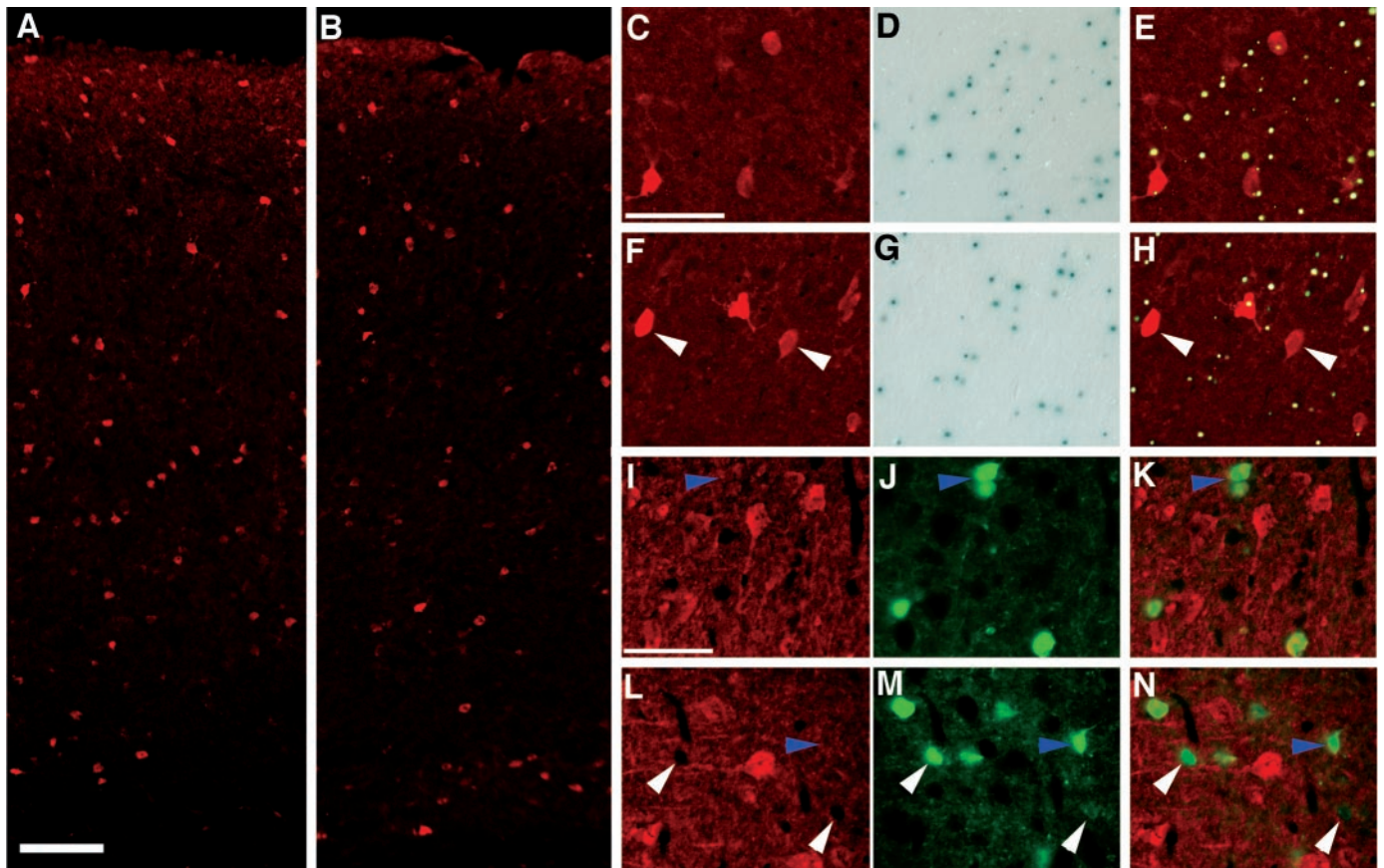


Figure 5. Cdk5-deficient GABAergic neurons are present within the cerebral cortex of Cdk5 chimeras. An essentially wild-type animal is shown in *A*, whereas a more balanced chimera is illustrated in *B*. Note the similar distribution of GABAergic cells in both animals. The remaining panels illustrate GABAergic immunostaining combined with X-gal histochemistry in wild-type (*C–E*) and Cdk5 chimeric (*F–H*) brains. Sections were stained with X-gal (*D, G*) and then were immunostained with anti-GABA antibodies (red; *C, F*). The X-gal staining (*D, G*) was digitally converted to green on a black background to allow overlay with GABA immunostaining for purposes of illustration (*E, H*). A green dot is associated with all GABAergic neurons in the wild-type chimera (*E*). In contrast, in Cdk5 chimeric mice (*H*), many GABAergic neurons are X-gal-negative (arrowheads), indicating that they are *cdk5*^{-/-}. To further confirm the presence of *cdk5*^{-/-} GABAergic neurons, wild-type (*I–K*) and Cdk5 (*L–N*) chimeras were double-labeled with antibodies to Cdk5 (*I, L*) and calbindin (*J, M*), a marker for a subpopulation of GABAergic neurons (Hendry et al., 1989; Van Brederode et al., 1990). The images were digitally combined (*K, N*) for clarity. In normal cortex (*I–K*), all GABAergic neurons are Cdk5-positive (blue arrowhead). In the chimeric cortex (*L–N*), although many of calbindin-positive neurons are also Cdk5-positive (blue arrowhead), there are substantial numbers of Cdk5-negative, calbindin-positive neurons (white arrowheads). Scale bars: *A, B*, 100 μ m; *C–N*, 50 μ m.

and C and other controls were not counted, because these animals contained only *cdk5*^{+/+} neurons.

Previous studies of cerebral cortex of *cdk5*^{-/-} embryos indicated that only the earliest born cortical neurons are capable of entering the cortical plate, whereas the rest remain trapped below

the subplate in the underplate (Gilmore et al., 1998). In Cdk5 chimeras, however, there are infrequent but regular examples of *cdk5*^{-/-} neurons found in layers II–V. We wished to determine the reason for this apparent discrepancy. Cortical plate neurons derive primarily from the ventricular zone of the dorsolateral

Table 3. Both wild-type and *cdk5*^{-/-} GABAergic neurons contribute to the cerebral cortex of Cdk5 chimeras

Mouse	β -Gal-negative (%)	β -Gal-positive (%)
423A	23	77
423B	7	93
423C	8	92
423D	40	60

The percentage of β -gal-negative neurons and β -gal-positive neurons is shown as a percentage of total GABAergic neurons counted from cerebral cortex, not including layer I and white matter tract. β -Gal status was determined as described in Figure 4 and Materials and Methods. The percentage of β -gal-negative neurons is low in the essentially wild-type chimeras 423B and 423C (see Table 1). In contrast, in higher mutant chimeras 423A and 423D, the number of β -gal-negative neurons increases dramatically. This indicates that *cdk5*^{-/-} neurons are contributing significantly to the GABAergic population of the cerebral cortex.

ventricle, but this is not their only source. The ganglionic eminences give rise to a large tangential migration of cells that are the likely precursors to the cortical GABAergic interneurons of cerebral cortex (de Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; for review, see Parnavelas, 2000). To determine whether *cdk5*^{-/-} cells in the upper layers of the chimeric cerebral cortex were these GABAergic neurons, immunostaining was performed with an anti-GABA antibody plus histochemical staining with X-gal for the genotype marker β -galactosidase. The spatial location of the GABA-positive neurons is the same in both wild-type mice (Fig. 5A) and all chimeras (Fig. 5B). An X-gal reaction product can be seen associated with most GABAergic neurons of the primarily wild-type chimera 423C (Fig. 5C–E), identifying them as *cdk5*^{+/+}. Chimera 423D (30% *cdk5*^{-/-}; Table 1) has many more GABAergic neurons

without associated X-gal staining (Fig. 5F–H, arrowheads). The presence of these GABA⁺/X-gal⁻ cells demonstrates that *cdk5*^{-/-} mutant GABAergic neurons survive and locate properly in the chimera. No pattern was noted in the mediolateral or anteroposterior location of β -gal-positive and -negative neurons, indicating that both *cdk5*^{+/+} and *cdk5*^{-/-} neurons are equally capable of migrating throughout the cerebral cortex.

To quantitate this effect, GABAergic neurons were counted and scored for the presence of X-gal precipitate. The results are shown in Table 3. The percentage of GABA⁺/X-gal⁻ neurons is 7% for 423B and 8% for 423C, 23% for 423A, and 40% for 423D. The essentially wild-type mice, 423B and C, having 7–8% GABA⁺/X-gal⁻, likely represent the false-negative rate, whereas the chimeric animals 423A and D have percentages that correspond approximately to the level of chimerism in these animals (Table 1). To confirm that *cdk5*^{-/-} GABAergic neurons exist within the Cdk5 chimeric cerebral cortex, calbindin and Cdk5 double immunostaining was performed (GABA and Cdk5 double immunostaining could not be performed because of antibody incompatibility). Calbindin labels a subset of GABAergic neurons within the cerebral cortex (Hendry et al., 1989; Van Bredereode et al., 1990; Gogelia and Hamori, 1992). In the essentially wild-type mouse, 423C, all calbindin-positive neurons were Cdk5-positive (Fig. 5I–K, blue arrowhead). However, whereas the chimeric mouse, 423D, also contained Cdk5-positive neurons (Fig. 5L–N, blue arrowhead), this animal also had numerous Cdk5-negative, calbindin-positive neurons (white arrowheads) in its cortex. This is additional evidence for the presence of Cdk5-negative GABAergic neurons within the cerebral cortex of this animal.

To confirm the ability of *cdk5*^{-/-} GABAergic neurons to migrate properly, we examined both wild-type and *cdk5*^{-/-} non-chimeric embryos. In E18.5 cerebral cortex, anti-GABA immunostaining is qualitatively distinct but quantitatively low compared with other brain regions (results not shown). This situation is true in both wild-type (Fig. 6A) and *cdk5*^{-/-} (Fig. 6B) animals. In E18.5 wild-type embryos, GABAergic neurons are found within deeper portions of the developing cerebral cortex [Fig. 6A, inset (magnified area of D), D] (Del Rio et al., 1992). GABAergic neurons are also found in the portion of the *cdk5*^{-/-} cortex that contains the early cortical plate (between the marginal zone and subplate, ~50 μ m below the pial surface; Gilmore et al., 1998) [Fig. 6B, inset (magnified area of D marked by arrowheads)]. These data validate the evidence from the Cdk5 chimeras that GABAergic neurons do not require Cdk5 activity for proper migration.

Our findings demonstrate that most *cdk5*^{-/-} neocortical neurons are deficient in their migration, but the complete status of wild-type neurons remains unknown. We have already shown that the chimeric cerebral cortex appears normal in its overall appearance and layering pattern (Fig. 3) and that it is composed of primarily wild-type neurons (Table 2, Fig. 4). It remains a possibility, however, that some *cdk5*^{+/+} neurons are disrupted in their migration by the presence mutant neurons during development. To investigate the migration of a particular population, the nucleoside analog BrdU was injected into pregnant dams at day 17 of gestation. Cortical neurons born at this time normally migrate to layers II and III in the mouse (Caviness, 1982; Nowakowski et al., 1989). Anti-BrdU immunostaining of adult wild-type animals treated in this way during their gestation reveals that most of the neurons labeled with BrdU are indeed found within layers II and

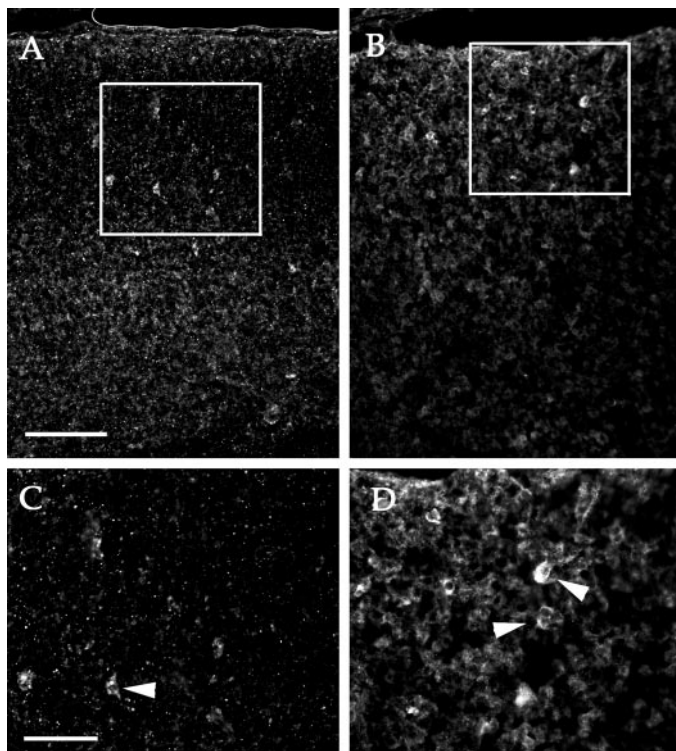


Figure 6. GABA staining is seen in both *cdk5*^{+/+} (A) and *cdk5*^{-/-} (B) E18.5 embryos. Insets in A and B are magnified in C and D, respectively. Arrowheads indicate locations of GABA-positive neurons in high-magnification images (C, D). Scale bars: A, B, 100 μ m; C, D, 50 μ m.

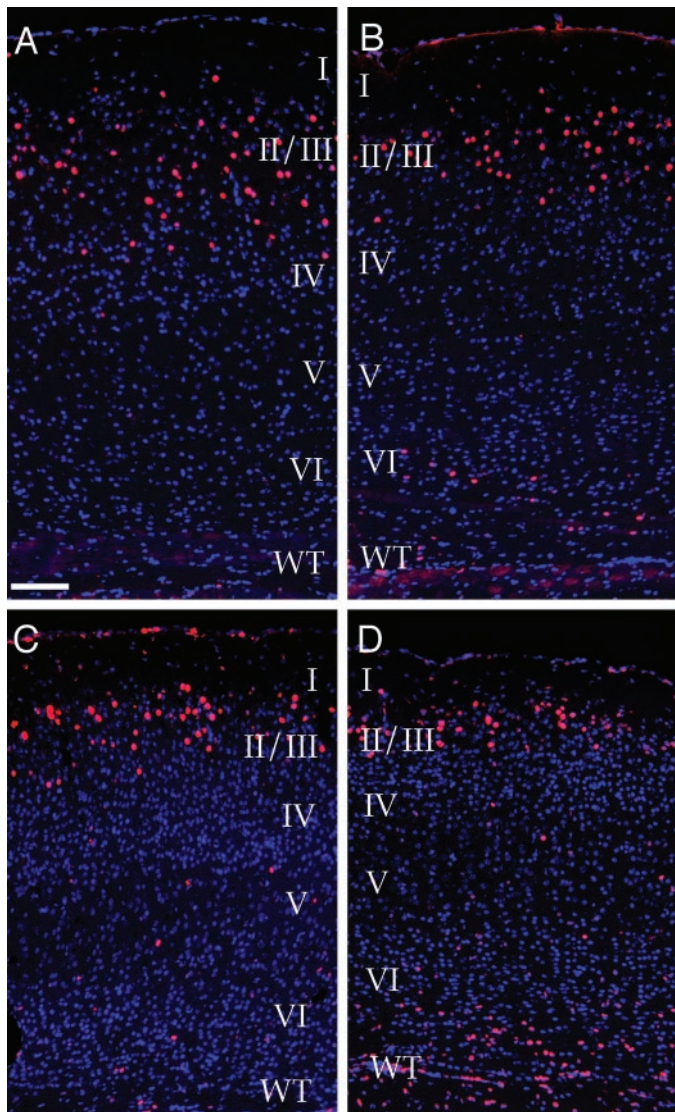


Figure 7. Migration of *cdk5*^{-/-} neurons is defective in the Cdk5 chimera. Wild-type (*A*, *C*) and chimeric (*B*, *D*) mice were labeled with BrdU at E17.5. Superimposed images of DAPI (blue) and anti-BrdU (red) staining are shown. BrdU-labeled neurons appear purple. In wild-type adult (*A*) and juvenile (postnatal day 12; *C*) mice, most of the E17-labeled neurons settle in layers II and III as expected (Caviness, 1982). In chimeras, many E17-labeled neurons end up in layers II and III, but a number are found deep to these layers as well. Similar results are found in both young (*D*) and old (*B*) animals. Note that small nuclei, faintly stained with BrdU, are present throughout the cortex. Because of their small size, they are likely to be glial cells. Scale bar, 100 μ m.

III (Fig. 7*A*). Interestingly, although many labeled neurons are also found in layers II and III in the chimera, many BrdU-labeled neurons remain in deep portions of cerebral cortex below layer VI (Fig. 7*B*) where *cdk5*^{-/-} neurons are found (Fig. 4, Table 2). A similar result was found in young (postnatal day 12) chimeras (Fig. 7*C,D*). Younger chimeras also display small, faintly stained BrdU-positive nuclei throughout the cerebral cortex. These nuclei are likely to belong to glial cells, because glial genesis begins approximately at the time of BrdU injection. We presume that the few large BrdU-labeled neurons not found within layers II and III or IV are likely to be GABAergic neurons, which are also born at this time (Miller, 1986).

DISCUSSION

The present analysis extends our previous studies of the defects in neuronal migration of Cdk5-deficient neurons. We find that neurons do not seem to require Cdk5 activity for simple migration activities *in vitro*. This is in contrast to other mutants in which sometimes subtle defects in migration *in vivo* display dramatic *in vitro* migration difficulties (Rezai and Yoon, 1972; Hatten et al., 1986; Reiner et al., 1993; Hirotsune et al., 1998). If Cdk5 deficiency worked in this manner, we would expect to find slower-migrating cells in our cultures from mutant cerebral cortex. Although it is theoretically possible that only Cdk5-independent neurons migrate away from the explants, we observed no difference between *cdk5*^{-/-} and wild-type explants in either the pattern or extent of cell movement. As a whole, our results suggest that Cdk5 is not an essential part of the basic movement machinery of a cell but instead plays a specific role in the much more complicated process of directed neuronal migration *in vivo*.

Chimeric animals are a powerful system in which to examine the adult cellular phenotypes of mutants that die during development and to determine how wild-type and mutant cells interact. Previous analysis of *cdk5*^{-/-} embryos demonstrated that early cortical plate precursors (layer VI) and preplate neurons do not require Cdk5 to migrate properly, whereas all other cortical plate neurons do (Gilmore et al., 1998). Analysis of the chimeras indicates that the Cdk5 deficiencies are cell-autonomous defects in migration. Most of the neurons in the cerebral cortex of the adult chimeras are wild-type, whereas most of *cdk5*^{-/-} cells are located ectopically at the bottom of the cerebral cortex. Examination of the cerebral cortex of Cdk5 chimeras also reveals the presence of mutant (*cdk5*^{-/-}) GABAergic neurons within the cerebral cortex. We conclude from this that Cdk5 activity is not required for proper migration of GABAergic neurons from ganglionic eminences into and throughout the cerebral cortex (de Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; for review, see Parnavelas, 2000).

Within *cdk5*^{-/-} mutant embryos, only the earliest born cortical neurons are capable of migrating through the subplate and separating it from the marginal zone (Gilmore et al., 1998). In the chimeras, many *cdk5*^{-/-} neurons are found mixed with their wild-type counterparts within the deep strata of layer VI. It is possible that some of these are true layer VI cells, because in both the mutant and the chimera they migrate past the subplate and separate it from the marginal zone, thus establishing a bona fide cortical plate. It is also possible that, during normal development, some neurons from the embryonic subplate survive into adulthood and become located in the deep portions of layer VI, as suggested by Landry et al. (1998). In addition, *cdk5*^{-/-} GABAergic neurons deep within the cortex can be confused with layer VI neurons. However, we think that our evidence supports the conclusion that the *cdk5*^{-/-} NeuN⁺ cells are true layer VI neurons for two reasons. First, some *cdk5*^{-/-} neurons are located in more superficial portions of this layer, an unlikely position for surviving subplate neurons. Second, in the mutant Cdk5-deficient embryos, there is a distinct population of layer VI-like neurons that separate the subplate from the marginal zone. Proper migration of *cdk5*^{-/-} layer VI neurons within Cdk5-deficient mice is in contrast to the situation in the *reeler* mouse, in which such cells are absent, and this crucial event in corticogenesis never occurs (Gilmore et al., 1998).

We have proposed previously, on the basis of the phenotype of the *cdk5*^{-/-} embryo, that layer VI neurons use a different mech-

anism of migration than do neurons in layers II–V (Gilmore et al., 1998). We suggested that layer VI neurons might use a Cdk5-independent mechanism to translocate their nucleus through a cytoplasmic process that remains attached to the pial surface, a mechanism that has been proposed previously (Morest, 1970; Nowakowski and Rakic, 1979; Brittis et al., 1995). We also speculated that radial glial-guided migration (Rakic, 1971a,b, 1972) might be used by the subsequent neurons of layers II–V. Recent studies by Nadarajah et al. (2001) using slice culture visualization of migration have found new evidence in support of this model. Early precursors appear to move their cell body in a manner consistent with nuclear translocation (Nadarajah et al., 2001). GABAergic neurons not only migrate from the ganglionic eminences and through the subventricular zone of the cerebral cortex, they must at some point more deeply penetrate the superficial cortical plate region of cerebral cortex. Because this process appears unaffected by the absence of Cdk5, GABAergic neurons must use different cues or different migrational mechanisms than layer II–V neurons to reach their final location.

The cell-autonomous requirement for Cdk5 shows some similarities to other mouse mutants. *Reeler* mice are deficient in the large extracellular protein reelin. This protein is thought to bind to several different extracellular receptors, resulting in the differential phosphorylation of the mouse disabled protein mDab1 (D'Arcangelo et al., 1995, 1997, 1999; Hirotsune et al., 1995; Ogawa et al., 1995; Howell et al., 1997, 1999; Sheldon et al., 1997; Ware et al., 1997; Rice et al., 1998; Hiesberger et al., 1999; Senzaki et al., 1999; Trommsdorff et al., 1999) (for review, see Rice and Curran, 1999; Gilmore and Herrup, 2000). Cerebellar Purkinje cells appear to require intrinsic Dab1, because all *Dab1*^{-/-} Purkinje cells in *Dab1* chimeras are blocked in their migration (Goldowitz, 1998). Correct migration would also appear to require a certain level of cell–cell interaction, however, because some wild-type Purkinje cells remain ectopically located in *Dab1* chimeras that are made up primarily of mutant cells (D. Goldowitz, personal communication). In addition, recent evidence demonstrates a genetic association between *Dab1* and the Cdk5 activator p35 (Ohshima et al., 2001). The exact relationship between reelin and *Dab1* and p35 and Cdk5 for proper migration is clearly a matter that requires further study.

The survival of substantial numbers of mutant cells in the mature chimeras indicates that Cdk5 deficiency per se is not toxic to neurons. GABAergic neurons of the cerebral cortex, Purkinje cells, deep cerebellar nuclear neurons, granule cells, and nearly all neurons in the embryo can survive without Cdk5, although precise quantitation has not been performed for any population (Gilmore et al., 1998; Ohshima et al., 1999). Nonetheless, in the chimera, *cdk5*^{-/-} neurons appear to be at a significant long-term survival disadvantage compared with wild-type neurons. Estimations of the percent chimerism from wild-type Purkinje cell counts can be used to illustrate that the percentage of *cdk5*^{-/-} neurons present in the cerebral cortex is much lower than would be predicted (Tables 1, 3). For 423A, the observed percentage is 15%, whereas the expected percentage is 32%; for 423D, 19% is the observed percentage, whereas 32% is expected. For chimera 326B, the numbers are even more dramatic. On the basis of the Purkinje cell counts, 40% of the cells of cerebral cortex should be mutant, yet we observed only 14% *cdk5*^{-/-} cells. The three animals combined have a lower than expected percentage of *cdk5*^{-/-} neurons that is statistically significant ($p < 0.005$). These calculations strongly suggest that *cdk5*^{-/-} neurons are selected against during development. It is noteworthy that the

counts of Cdk5-deficient neurons include the GABAergic neurons present in each field; therefore, the deficiency of neurons derived from the telencephalic ventricular zone is most likely even greater than these approximate calculations would imply. The missing cells cannot be explained by a deficit in the generation of *cdk5*^{-/-} neurons, because no detectable difference in the size of cerebral cortex is noted between *cdk5*^{-/-} and wild-type embryos at E18.5 (Ohshima et al., 1996; Gilmore et al., 1998).

Why *cdk5*^{-/-} neurons should be at such a competitive developmental disadvantage is not clear. Ectopic location alone does not prevent a neuron from finding a target. Significant neuronal ectopia has been observed in other mouse mutants such as *reeler*, *scrambler* (*dab1*^{-/-}), and the null allele of the Cdk5 activator p35; these displacements have no reported effect on cell survival within the cerebral cortex. In addition, characterization of the *reeler* mutant has shown repeatedly that mispositioned neurons are fully capable of finding their correct target (Lemmon and Pearlman, 1981; Simmons et al., 1982; Caviness and Frost, 1983; Frost et al., 1986) even if the time course of connectivity is delayed compared with wild type (Yuasa et al., 1994; Del Rio et al., 1997). If ectopic *cdk5*^{-/-} neurons were delayed in finding a target in chimeras, that could put them at a competitive disadvantage compared with wild type. This situation may be amplified further, because Cdk5 has been suggested to be important for normal axonal outgrowth and synaptogenesis (Matsubara et al., 1996; Nikolic et al., 1996, 1998; Xiong et al., 1997; Paglini et al., 1998; Qi et al., 1998; Shuang et al., 1998; Fletcher et al., 1999). Thus, lack of survival of *cdk5*^{-/-} neurons could be attributable to migrational, axonal, or synaptic defects, or to an interaction of all three.

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